

# Dichloro-phenyl-benzotriazoles: a new selective class of Human Respiratory Syncytial virus entry inhibitors

Sandra Piras<sup>a</sup>, Giuseppina Sanna<sup>b</sup>, Antonio Carta<sup>a,\*</sup>, Paola Corona<sup>a</sup>, Roberta Ibba<sup>a</sup>, Roberta Loddo<sup>b,\*</sup>, Silvia Madeddu<sup>b</sup>, Paola Caria<sup>b</sup>, SuzanaAulic<sup>c</sup>, Erik Laurini<sup>c</sup>, Maurizio Fermeglia<sup>c</sup>,  
Sabrina Prici<sup>c</sup>.

<sup>a</sup> Department of Chemistry and Pharmacy, University of Sassari, IT, Italy.

<sup>b</sup> Department of Biomedical Sciences, University of Cagliari, IT, Italy.

<sup>c</sup> Molecular Simulation Engineering (MOSE) Laboratory, University of Trieste, IT, Italy.

**TABLE S1.** Activity of 5,6-dichloro-1-phenyl-benzotriazole amides(**5a-d** and **7a-h**) against viruses representative of positive-sense, single-stranded RNAs (ssRNA+): i) Retroviridae: HIV-1; ii) Flaviviridae: YFV and BVDV; iii) Picornaviridae: CV-B5 and Sb-1. Viruses representative of negative-sense, single-stranded RNAs (ssRNA-); i) Rhabdoviridae: VSV. Virus representative of double-stranded RNAs (dsRNA): Reoviridae: Reo-1. DNA virus representatives: i) Poxviridae: VV; ii) Herpesviridae: HSV-1. Efavirenz, 2'-C-methyl-guanosine, and Pleconaril were used as reference inhibitors. Data represent mean values  $\pm$  SD for three independent determinations. For values where SD is not shown, variation among duplicate samples was less than 15%. Efavirenz (EFV), 2'-C-methyl-guanosine (2MG), and Pleconaril (PCL) were used as reference inhibitors.

Cpd	MT-4 cells	HIV-1 <sub>IIIb</sub>	MDBK cells	BVDV	BHK cells	YFV	Reo-1	Vero-7 6 cells	CV-B5	Sb-1, VSV, VV, HSV-1
	CC <sub>50</sub> <sup>a</sup>	EC <sub>50</sub> <sup>b</sup>	CC <sub>50</sub> <sup>c</sup>	EC <sub>50</sub> <sup>d</sup>	CC <sub>50</sub> <sup>e</sup>	EC <sub>50</sub> <sup>f</sup>	EC <sub>50</sub> <sup>g</sup>	CC <sub>50</sub> <sup>h</sup>	EC <sub>50</sub> <sup>i</sup>	EC <sub>50</sub> <sup>j</sup>
<b>1a</b>	>100	>100	>100	<b>75</b>	>100	>100	>100	>100	85	>100
<b>5a</b>	35	>35	43	>43	53	>53	>53	30	<b>17</b>	>30
<b>5b</b>	28	>28	>100	>100	54	>54	>54	30	>30	>30

<b>5c</b>	60	>60	>100	>100	>100	>100	>100	10	<b>9</b>	>100
<b>5d</b>	35	>35	14	>14	16	>16	>16	20	>20	>20
<b>7a</b>	>100	>100	>100	>100	44	>44	>44	>100	>100	>100
<b>7b</b>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<b>7c</b>	33	>33	>100	>100	>100	>100	>100	>100	>100	>100
<b>7d</b>	77	>77	>100	>100	>100	<b>78</b>	>100	90	>90	>90
<b>7e</b>	>100	>100	>100	>100	96	>96	>96	>100	>100	>100
<b>7f</b>	>100	>100	>100	>100	84	>84	>84	>100	>100	>100
<b>7g</b>	>100	>100	>100	>100	>100	>100	>100	9	>90	>90
<b>7h</b>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<b>Ref Cpd</b>										
<b>EFV</b>	40	0.002 ± 0.0002								
<b>2MG</b>			>100	1.1 ± 0.1	>100	1.9 ± 0.1	0.7 ± 0.2			
<b>PCL</b>								>10 0	0.005 ± 0.001	

<sup>a</sup>Compound concentration (μM) required to reduce the proliferation of mock-infected MT-4 cells by 50%, as determined by the MTT method. <sup>b</sup>Compound concentration (μM) required to achieve 50% protection of MT-4 cells from HIV-1 induced cytopathogenicity, as determined by the MTT method. <sup>c</sup>Compound concentration (μM) required to reduce the viability of mock-infected MDBK cells by 50%, as determined by the MTT method. <sup>d</sup>Compound concentration (μM) required to achieve 50% protection of MDBK cells from BVDV-induced cytopathogenicity, as determined by the MTT method. <sup>e</sup>Compound concentration (μM) required to reduce the viability of mock-infected BHK cells by 50%, as determined by the MTT method. <sup>f</sup>Compound concentration (μM) required to achieve 50% protection of BHK cells from YFV-induced cytopathogenicity, as determined by the MTT method. <sup>g</sup>Compound concentration (μM) required to achieve 50% protection of BHK cells from Reo-1-induced cytopathogenicity, as determined by the MTT method. <sup>h</sup>Compound concentration (μM) required to reduce the viability of mock-infected VERO-76 cells by 50%. as determined by the MTT method. <sup>i</sup>Compound concentration (μM) required to reduce the plaque number of CV-B5 by 50% in VERO-76 monolayers. <sup>j</sup>Compound concentration (μM) required to reduce the plaque number of Sb-1, VSV, VV and HSV-1 by 50% in VERO-76 monolayers.

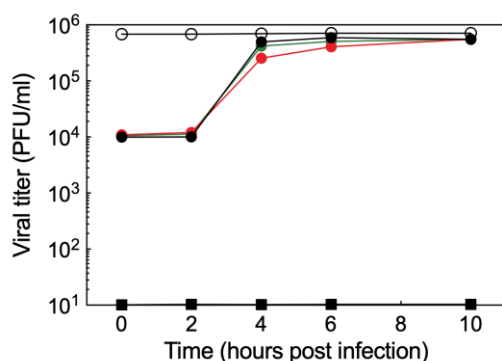
**TABLE S2.** Activity of 5,6-dichloro-2-phenyl-benzotriazole amides(**6a-h** and **8a-h**), and 5,6-dichloro-2-phenyl-benzotriazole urees(**10a-k**) against viruses representative of positive-sense, single-stranded RNAs (ssRNA+): i) Retroviridae: HIV-1; ii) Flaviviridae: YFV and BVDV; iii) Picornaviridae: CV-B5 and Sb-1. Viruses representative of negative-sense, single-stranded RNAs (ssRNA-); i) Rhabdoviridae: VSV. Virus representative of double-stranded RNAs (dsRNA): Reoviridae: Reo-1. DNA virus representatives: i) Poxviridae: VV; ii) Herpesviridae: HSV-1. For values where SD is not shown, variation among duplicate samples was less than 15%.Efavirenz (EFV), 2'-C-methyl-guanosine (2MG), and Pleconaril (PCL) were used as reference inhibitors.

Cpd	MT-4 cells	HIV-1 <sub>IIIb</sub>	MDBK cells	BVDV	BHK cells	YFV	Reo-1	Vero-76 cells	CV-B5	Sb-1, VSV, VV, HSV-1
	CC <sub>50</sub> <sup>a</sup>	EC <sub>50</sub> <sup>b</sup>	CC <sub>50</sub> <sup>c</sup>	EC <sub>50</sub> <sup>d</sup>	CC <sub>50</sub> <sup>e</sup>	EC <sub>50</sub> <sup>f</sup>	EC <sub>50</sub> <sup>g</sup>	CC <sub>50</sub> <sup>h</sup>	EC <sub>50</sub> <sup>i</sup>	EC <sub>50</sub> <sup>j</sup>
<b>1b</b>	52	>52	≥100	20	>100	>100	>100	>100	>100	>100
<b>6a</b>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<b>6b</b>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<b>6c</b>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<b>6d</b>	33	>33	100	>100	72	>72	>100	>100	>100	>100
<b>6e</b>	15	>15	72	>72	26	>26	>26	>100	<b>61</b>	>100
<b>6f</b>	24	>24	84	>84	62	>62	>26	>100	<b>33</b>	>100
<b>8a</b>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<b>8b</b>	>100	>100	>100	>100	>100	<b>73</b>	>100	>100	>100	>100
<b>8c</b>	>100	>100	>100	>100	>100	>100	>100	>100	<b>14</b>	>100
<b>8d</b>	63	>63	>100	<b>35</b>	35	>35	>35	80	>80	>80
<b>8e</b>	>100	>100	>100	<b>4</b>	68	>68	>68	>100	>100	>100
<b>8f</b>	>100	>100	>100	<b>60</b>	>100	>100	>100	80	>100	>100

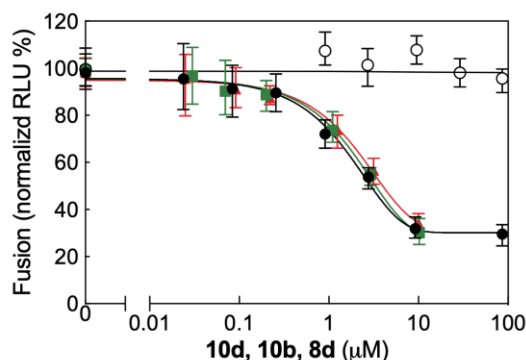
<b>8g</b>	>100	>100	>100	<b>28</b>	>100	>100	>100	>100	>100	>100
<b>8h</b>	>100	>100	>100	<b>11</b>	>100	>100	>100	>100	>100	>100
<b>10a</b>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<b>10b</b>	>100	>100	78	>78	40	>40	>40	30	>30	>30
<b>10c</b>	>100	>100	>100	>100	>100	>100	>100	90	>95	>95
<b>10d</b>	>100	>100	>100	>100	71	>71	>71	90	>90	>90
<b>10e</b>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<b>10f</b>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<b>10g</b>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<b>10h</b>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<b>10i</b>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<b>10j</b>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<b>10k</b>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<b>Ref Cpd</b>										
<b>EFV</b>	40	0.002 ± 0.0002								
<b>2MG</b>			>100	1.1 ± 0.1	>100	1.9 ± 0.1	0.7 ± 0.2			
<b>PCL</b>								>100	0.005 ± 0.001	

<sup>a</sup>Compound concentration (μM) required to reduce the proliferation of mock-infected MT-4 cells by 50%, as determined by the MTT method. <sup>b</sup>Compound concentration (μM) required to achieve 50% protection of MT-4 cells from HIV-1 induced cytopathogenicity, as determined by the MTT method. <sup>c</sup>Compound concentration (μM) required to reduce the viability of mock-infected MDBK cells by 50%, as determined by the MTT method. <sup>d</sup>Compound concentration (μM) required to achieve 50% protection of MDBK cells from BVDV-induced cytopathogenicity, as determined by the MTT method. <sup>e</sup>Compound concentration (μM) required to reduce the viability of mock-infected BHK cells by 50%, as determined by the MTT method. <sup>f</sup>Compound

concentration ( $\mu\text{M}$ ) required to achieve 50% protection of BHK cells from YFV-induced cytopathogenicity, as determined by the MTT method. <sup>e</sup>Compound concentration ( $\mu\text{M}$ ) required to achieve 50% protection of BHK cells from Reo-1-induced cytopathogenicity, as determined by the MTT method. <sup>b</sup>Compound concentration ( $\mu\text{M}$ ) required to reduce the viability of mock-infected VERO-76 cells by 50%, as determined by the MTT method. <sup>i</sup>Compound concentration ( $\mu\text{M}$ ) required to reduce the plaque number of CV-B5 by 50% in VERO-76 monolayers. <sup>j</sup>Compound concentration ( $\mu\text{M}$ ) required to reduce the plaque number of Sb-1, VSV, VV and HSV-1 by 50% in VERO-76 monolayers.



**Figure S1.** Inhibition of RSV (m.o.i = 1) by addition of 20  $\mu\text{M}$  of compound **10d** (black filled circles), **10b** (green filled circles), and **8d** (red filled circles) at different times. Data for untreated virus (open circles) and for addition of 6-azauridine (filled squares) are also shown for comparison. Data represent mean values from two independent determinations; variation among duplicate samples was less than 15%. Data for **10b** and **8d** were obtained under the same conditions employed for **10d** (see main text, Materials and Methods section).



**Figure S2.** Quantitative dose-response cell-to-cell fusion assay using the DSP-chimeric reporter proteins and the ViviRenrenilla luciferase substrate in the presence of compounds **10d** (black filled symbols), **10b** (green filled symbols), and **8d** (red filled symbols). The MeV (Measles Virus) F and H glycoprotein expression constructs (open symbols) were included for selectivity control. Reported values are normalized for DMSO-treated samples and are expressed as the mean of three experiments  $\pm$  standard deviation. The  $\text{EC}_{50}$  values for the three compounds, obtained by 4-parameter variable slope regression fitting, are: 3.2  $\mu\text{M}$  for **10d**, 3.9  $\mu\text{M}$  for **10b**, and 4.5  $\mu\text{M}$  for **8d**, respectively. Data for **10b** and **8d** were obtained under the same conditions employed for **10d** (see main text, Materials and Methods section).